Connective Tissue Activation XXXVIII: Heparin/Heparanase Activity of Human Platelets Resides in a High Molecular Weight Protein, Not in Connective Tissue Activating Peptide III

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ABSTRACT ***Objective.*** Connective tissue activating protein-III (CTAP-III), with molecular weight 9278 Da and isoforms including CTAP-III des 1–15 (neutrophil activating peptide-2, NAP-2) and other amino terminal deletion isoforms, has been isolated from human platelets and characterized. Platelets have also been shown to possess significant heparin/heparanase activity. We investigated whether human platelet heparin/heparanase activity derives from CTAP-III.

Methods. Radial immunodiffusion measurement showed substantial amounts of CTAP-III in plasma from outdated platelet packs. A convenient method for measurement of heparin/heparanase activity is described, and with this method platelet associated plasma was investigated for heparin/heparanase activity assayed against ^{3}H-heparin and ^{35}S-heparan sulfate.

Results. Removal of CTAP-III from platelet associated plasma with an immunospecific immunoaffinity column did not remove the heparin/heparanase activity from the plasma. Highly purified CTAP-III eluted from an immunospecific affinity column lacked heparin/heparanase activity.

Conclusion. Human platelet heparin/heparanase activity resides not in CTAP-III but in a protein or proteins with molecular weight ≥ 55 kDa. (J Rheumatol 2002;29:2337–44)

Key Indexing Terms:
HEPARIN/HEPARANASE ACTIVITY CONNECTIVE TISSUE ACTIVATING PEPTIDE-III PLATELET FACTOR-4

Connective tissue activating peptide-III (CTAP-III), a chemokine with molecular weight 9278 Da and isoforms including CTAP-III des 1–15 (neutrophil activating peptide-2, NAP-2) and several other amino terminal deletion isoforms, has been isolated from human platelets and characterized[1-5]. Platelets have also been shown to possess significant heparin/heparanase activity that cleaves heparin and heparan sulfate into their respective oligosaccharide constituents[6]; preparations purified 300-fold retained activity against both substrates. A study of human platelet heparinase/heparanase suggested that the enzyme had a molecular weight of 134 kDa and cleaved heparin over a pH range from 4 to 9[7]. In the past 5 years, 2 laboratories identified CTAP-III as a platelet derived heparin/heparanase[8,9]. Three laboratories have published data indicating that platelet derived heparin/heparanase enzymatic activity resides in a protein with a molecular weight in the range of 34 to 134 kDa[10-12]. A recent review supported the idea that CTAP-III is a heparinase/heparanase[13].

Our studies show that heparin/heparanase activity of platelet-rich plasma supernatant resides not in the protein fraction binding to a CTAP-III immunoaffinity column, but among those proteins that do not bind to this column, and in which CTAP-III is not detectable. While it is clear that CTAP-III occurs in many isoforms, the precise characteristics of which may vary in different preparations and as a function of time, they all react with anti-CTAP-III antibody[14]. In view of the significant homology between CTAP-III and platelet factor-4 (PF-4), we assessed the heparinase enzyme activity of both these low molecular weight platelet proteins.

MATERIALS AND METHODS

rPF-4 was kindly provided by the Repligen Corporation, Waltham, MA, USA. Antibodies included rabbit anti-rCTAP-III raised against Leu 21 rCTAP-III (rabbit anti-human PF-4; Celsus Laboratories, Cincinnati, OH, and Chemicon International Inc., Temecula, CA, USA). Rabbit antisera raised against native human PF-4 was obtained from Accurate Chemical and Scientific Corp., Westbury, NY, USA. [^{3}H]-Heparin was obtained from

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Dupont-New England Nuclear; glycosaminoglycan cleaving enzymes ABCase, heparin lyase I (EC 4.2.2.7), heparin lyase II, and heparinase III (heparitinase, EC 4.2.2.8) from *Flavobacterium heparinum* were purchased from Sigma Chemical Co., St. Louis, MO, USA. rCTAP-III-Leu-21 was prepared as described[4].

Column fractionalization utilized Sephacryl S-200 HR from Pharmacia, Peapack, NJ, USA. Heparin affinity chromatography procedures utilized columns from Amersham-Pharmacia Biotech, Piscataway, NJ, USA.

Measurement of heparinase/heparanase activity. This method of identification and measurement of enzyme activities that degrade specific glycosaminoglycans (GAG) depends on the insolubility of cetylpyridinium chloride (CPC)-GAG complexes when the GAG are in a polymerized state and their solubility when depolymerized. In the latter circumstance depolymerized GAG are easily washed from Whatman 3 MM chromatography paper and the amount of labeled substrate that has been released as a function of enzyme activity can then be determined by difference[14] A similar assay method was described by Oldberg, *et al* in their initial description of platelet heparin/heparan degrading enzyme activity[6]. We confirmed that this method distinguishes between polymerized and smaller GAG subunits by subjecting [^{3}H]-heparin, suitable controls, and the ^{3}H labeled substrate treated with heparinase I to centrifugation at 6500 g in Centricon microconcentrators 3 and 10, which have 3 and 10 kDa molecular weight cutoffs, respectively. Heparinase I clearly reduced the retent and increased the filtered fraction of [^{3}H]-heparin to the same degree as observed with the more convenient CPC procedure described in detail below.

Enzyme assays were performed in quadruplicate in Costar 96 half-well flatbottom plastic tissue culture plates. Polymeric GAG form insoluble precipitates with CPC in the presence of 0.3 ml NaCl, whereas enzymatically digested GAG are soluble under these conditions. To measure heparinase activity, 25 μl of sodium [^{3}H]-heparin served as substrate at a final concentration of 0.05 μCi/well. Low molecular weight ^{3}H labeled components were removed by extensive dialysis against distilled water with a 1 kDa molecular weight cutoff dialysis casing prior to use. For measurement of heparanase activity, [^{35}S]-heparan sulfate isolated from human umbilical vein endothelial cell cultures served as substrate. The enzymes were added in 30 μl volumes to each well of Costar 96 half-well plates. These included heparin lyase I, heparin lyase II, and heparin lyase III. The pH was adjusted according to the requirements of the various enzymes; pH 7.5 in the case of heparin lyase I. Putative enzyme preparations of platelet origin were added in 30 μl volumes to each well and the reaction mixtures adjusted to pH 6.0, a pH value found to yield maximal enzyme activity.

The 96 well plates were closed and incubated at 37°C for 16–18 h in a humidified chamber. On conclusion of heparin/heparan digestion, 10.0 μg of Pronase in 33 μl of 20 mM Tris buffer containing 50 ml NaCl at pH 8.3 was added, bringing the incubation volume to 88 μl. Assay plates were then incubated at 37°C for 2 h to permit proteolytic digestion of the incubation mixture. This step is essential to avoid binding of the GAG substrate to the plastic well. Twenty-two microliters of 20 mM Tris, 3 M NaCl adjusted to pH 8, were added to each reaction mixture to a final volume of 110 μl.

The final NaCl concentration in each well was 0.64 M. Incubation at room temperature for 30 min prevented nonspecific binding of labeled substrate to the plastic wells. The contents of each well were then spotted onto 3 MM Whatman chromatography paper, previously marked with 4×4 cm squares. The paper sheets were dried at 37°C for at least 2 h. Each paper sheet was then washed 4 times for 30 min each, in 0.1% CPC containing 0.3 M NaCl, and then dried at 37°C. The areas containing the GAG-CPC precipitates were excised, folded, and placed into liquid scintillation vials containg 5 ml of Ecolite (ICN 882475) liquid scintillation cocktail. The isotope content of the residual carbohydrate components was measured in a Beckman LS 7000 scintillation spectrometer. Enzyme activity is expressed as the percentage digestion of the carbohydrate substrate, i.e., the percentage of the GAG precipitate rendered soluble and consequently washed off the chromatography paper. Enzyme activity was linear as a function of protein concentration and incubation time.

Isolation of CTAP-III from frozen platelets. A modification of our procedure for isolating CTAP-III from frozen outdated human platelets reduced the time consumed in Sephacryl and heparin column chromatography steps[4]. Unless stated otherwise, all steps were conducted at 4°C. Fifty grams, wet weight, of outdated human platelets were extracted overnight with 500 ml of an HCl/ethanol mixture (5 ml 1.25 M HCl and 95 ml ethanol). The preparation was then centrifuged 20 min at 10,000 g. Three volumes of acetone were added to the supernatant, stirred for 1 h, and allowed to stand for an additional hour. The supernatant fluid was aspirated and the precipitate dissolved in 50 ml of 0.5 M acetic acid and dialyzed against 150 mM Dulbecco's phosphate buffered saline (DPBS), pH 7.0, for 48 h with one change of the dialysis bath. The resulting precipitate was removed by centrifugation for 1 h at 10,000 g. The supernatant was then further fractionated using a Minitan ultrafiltration system, which retains proteins with a molecular weight > 30 kDa (Centricon-30; Amicon, Bedford, MA, USA) to remove the bulk of the larger proteins. The filtrate was concentrated in a Centricon-3 kDa microconcentrator. About 75% of the protein was found in the 30 kDa retent. Nearly all of the remaining protein was in the 3 kDa fraction. The 3 kDa retent was passed over an immunoaffinity column containing monospecific rabbit IgG (anti-CTAP-III) covalently linked to protein-A Agarose (Bio-Rad, 153–6153)[4]. After washing with PBS, bound CTAP-III was eluted with 0.1 M acetic acid (pH 2.8).

CTAP-III des 1–15 (NAP-2) was prepared from CTAP-III by cleavage with chymotrypsin at 10°C for 90 min, followed by heat inactivation of the product. CTAP-III des 1–15 (NAP-2) was isolated using the CTAP-III immunoaffinity column.

Protein measurement. Protein content was measured by a spectrophotometric method[15], except in the case of CTAP-III, where a radial immunodiffusion assay was used.

Cell culture methods. Human umbilical cord endothelial cells (HUVEC) were obtained from the American Type Culture Collection (CRL-1730) and cultured in monolayers. Culture medium consisted of F-12K medium supplemented with 2 mM *l*-glutamine and 1.5 g sodium bicarbonate/l. Additional medium supplements included heparin, 100 μg/ml, endothelial cell growth supplement, 30 μg/ml (E-0760), and 10% fetal bovine serum. All culture medium components were from Sigma, with the exception of the fetal bovine serum (Intergen Corp., Norcross, GA, USA) and sodium bicarbonate (Mallinckrodt, Phillipsburg, NJ, USA). The HUVEC cells were grown in T-25 tissue culture flasks with 5 ml of medium; incubations were carried out at 37°C in 5% CO_2. At confluency, cells were transferred to 75 cm^2 flasks.

Preparation of [$^3SO_4$] heparan sulfate. To test human platelet derived chemokines for heparanase activity, [$^{35}SO_4$] labeled human heparan sulfate was isolated from medium supporting the growth of HUVEC and from tryptic digests of the cells. When HUVEC reached confluent growth in 75 cm^2 flasks, the growth medium was removed and the flask rinsed with sterile DPBS. To label the GAG, fresh medium containing $^{35}SO_4$ (50 μCi/ml) was added to the endothelial cell cultures and incubated at 37°C for 24–72 h in 5% CO_2. Chondroitin sulfates A, B, and C and heparin were removed by incubation at 37°C for 24 h with chondroitinase ABC, 2 units/5 ml, and heparinase I, 8 units/5 ml, followed by heat inactivation at 90°C for 3 min. The residual heparan sulfate was concentrated by dialysis against 15% polyethylene glycol (MW 10,000) at 8°C in distilled water using membranes with a 1 kDa cutoff to remove the products of enzyme digestion. Sterilization was achieved by filtration through a 0.22 μm filter and the product stored at 4°C. The residual $^{35}SO_4$ labeled GAG was shown to be heparan sulfate by its sensitivity to heparanase III. Virtually none of the radioactive preparation was able to pass through a 30 kDa cutoff microconcentrator

Gel electrophoresis. Gel electrophoresis of protein samples was carried out using the NuPage Electrophoresis system. Protein samples isolated from platelets, and appropriate molecular weight standards, were visualized with silver staining with the Novex Silver x-Press kit (LC 6100).

Heparin affinity columns. One milliliter HiTrap Heparin columns were obtained from Amersham Pharmacia.

RESULTS

CTAP-III lacks heparanase/heparinase activity. Five units of outdated human platelets and associated plasma were centrifuged at 10°C for 1 h at 5000 g. One hundred fifty milliliters of the supernatant were then concentrated by dialysis against 2 l of 15% PEG at 10°C for 24 h using a 3.5 kDa MW cutoff dialysis membrane. The concentrated preparation was centrifuged at 10°C for 3 h at 5000 g and then passed through a 0.45 μm Millipore filter. The concentrated plasma preparation had a protein concentration of 101 mg/ml and 110 μg/ml of CTAP-III, respectively, as measured by radial immunodiffusion employing anti-CTAP-III antibodies. This preparation was then passed 3 times over an immunoaffinity column prepared with rabbit antibodies against CTAP-III. The data shown in Tables 1 and 2 show that the fraction that did not bind to the CTAP-III immunoaffinity column contained no detectable CTAP-III, but had the capacity to digest ^{35}S labeled heparan sulfate and [^{3}H]-heparin. Materials that bound to the immunoaffinity column were shown by radial immunodiffusion to contain 300 μg/ml of CTAP-III/ml; this fraction was also devoid of heparinase/heparanase activity.

Heparinase/heparanase activity in fresh and outdated human platelets. The fact that heparanase/heparinase activity associated with outdated platelets was found in the accompanying plasma raised the question of whether the enzyme is found in platelet associated plasma during the period when platelets are used clinically. To answer this question, platelets and the associated plasma from 2 and 5-day-old platelet packs were assayed separately. Heparinase activity was found only in extracts of the washed platelets. Assays showed that incubation of radioactive heparin with 53 μg of protein from a crude extract of washed platelets resulted in 23% hydrolysis of the substrate over an 18 h period. In contrast, 48 μg of the plasma proteins previously associated with the fresh platelets had no measurable heparanase activity. Since platelet packs are discarded as outdated at the end of Day 5, it seems clear that no significant leakage of the heparinase into the plasma occurs during the period when the platelets are used for clinical purposes.

Table 1. Hydrolysis of ^{35}S-heparan sulfate by a fraction of platelet-rich plasma that does not bind to a CTAP-III immunoaffinity column.

Sample	pH	^{35}S-Heparan Sulfate, cpm ± SD	Hydrolysis % (t > 10)
Controls			
20 mM Tris buffer	7.5	989 ± 23	0
Heparanase I, 0.2 units	7.5	755 ± 40	24
Heparanase III, 0.2 units	7.5	496 ± 16	50
Control buffer, 20 mM Tris with 10 mM Ca^{++} and Mg^{++}	6.0	809 ± 33	0
Reaction mixtures			
Platelet supernate, 2.0 mg protein/well	6.0	590 ± 61	27
Protein that bound to CTAP-III IMA column, 52 μg/well	6.0	868 ± 53	0
Protein not bound to CTAP-III IMA column, 527 μg/well	6.0	555 ± 31	31

Table 2. Hydrolysis of ^{3}H-heparin by a fraction of platelet-rich plasma that does not bind to a CTAP-III immunoaffinity column.

Sample	pH	^{3}H-Heparin, cpm ± SD	% Hydrolysis (t > 14)
Controls			
20 mM Tris	7.5	2109 ± 126	0
Heparinase I, 0.2 units	7.5	442 ± 22	79
20 mM Tris with 10 mM Ca^{++} and Mg^{++}	6.0	2162 ± 45	0
Reaction mixtures			
Platelet supernate, 2.0 mg/well	6.0	1473 ± 50	32
Platelet supernate, 52 μg/well, bound to and eluted from CTAP-III IMA column	6.0	2108 ± 61	0
Platelet supernate that did not bind to CTAP-III IMA column, 527 μg/well	6.0	1569 ± 74	27

Stability of heparinase activity in outdated platelets. Washed, outdated human platelets stored at –70˚C for as long as 7 years still exhibited substantial heparinase activity that was quantitatively indistinguishable from that of fresh platelets.

Extraction of heparanase/heparinase activity from outdated human platelets. Thirty to 35 units of platelets were pelleted by centrifugation, and washed 3 times at 4˚C with roughly 150 ml of 0.05 M phosphate buffer, pH 7.0, containing 0.01% EDTA. Washed platelets were then collected by centrifugation at 5000 g for 30 min before freezing and storage at –80˚C.

Extraction of heparin/heparanase enzyme activity from frozen outdated human platelets was accomplished at 4˚C by either (1) overnight extraction with 0.02 M Tris buffer, containing 50 mM NaCl, 10 mM CaCl and MgCl, at pH 6.0; or (2) by immediate sonication for 30 min and removal of insoluble material by centrifugation at 10,000 g for 1 h. Enzyme activity measurements showed that the amount of heparinase activity per mg of total extracted protein was essentially the same by each method. We found it most convenient to use the latter procedure. Concentration of protein in crude platelet extracts was accomplished by centrifuging the extract through a Centricon 30 kDa microconcentrator at 5000 g for 90 min. This step resulted in partial loss of CTAP-III, but did not result in loss of enzyme activity.

Ammonium sulfate fractionation. Crude platelet extract was dialyzed at 8˚C overnight against a range of $(NH_4)_2SO_4$ concentrations. Concentrations from 20 to 50% $(NH_4)_2SO_4$ saturation contained the major portion of the enzyme activity, but the enzyme was not cleanly resolved into either the precipitate or supernatant fractions. The complexity of ammonium sulfate fractions from 2 different crude platelet extracts was confirmed by gel electrophoresis (Figure 1). Lanes "g" and "h" illustrate the similarities and differences seen in protein mixtures extracted from different batches of outdated frozen platelets. The heparin/heparanase activity measured in each of the fractions is presented in Table 3.

Modest, but significant, enzyme activity (11% hydrolysis) was found in the protein fraction precipitated from preparation H-8 by 25% $(NH_4)_2SO_4$. Gel electrophoresis showed that this enzymatically active fraction (Figure 1, lane "e") contained only proteins ranging in molecular weight from 55 to 100 kDa.

Sephacryl G-200 column fractionation. A 2.0 × 27 cm Sephacryl S-200 HR column was prepared and calibrated separately with Dextran Blue, bovine serum albumin, and cytochrome C to define the void volume and the elution volume of the high and low molecular weight fractions, respectively[1]. CTAP-III is known to elute with cytochrome C on this column[1]. Crude platelet extract, concentrated in a Centricon 30 microconcentrator, was applied to the Sephacryl G-200 column and 7 fractions were obtained.

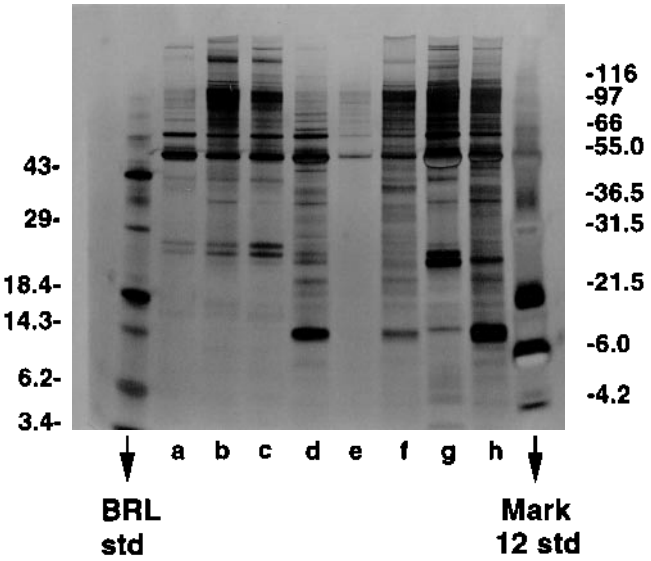

Figure 1. SDS gel of crude platelet extracts H-7 and H-8 and ammonium sulfate fractions. (a) H-7 50% supernatant; (b) H-7 40% precipitate; (c) H-7 50% precipitate; (d) H-8 50% supernatant; (e) H-8 25% precipitate; (f) H-8 50% precipitate; (g) H-7 crude extract; (h) H-8 crude extract.

Enzyme activity was found primarily in fraction 1, suggesting that the protein(s) containing heparinase/heparanase activity is of a high molecular weight; little enzyme activity was found in fraction 2, and none was detected in the fraction where cytochrome C eluted (Table 4, Figure 2).

Fractions 1 and 2 were each subjected to further fractionation with 40% and 50% $(NH_4)_2SO_4$. The data shown in Figure 2 and Table 5 indicate clearly that platelet heparinase activity resides in a high molecular weight species ranging from 55 to 200 kDa. The results presented in Table 5 show that the 50% $(NH_4)_2SO_4$ supernate of the mixture of proteins present in G-75 fraction 1 had a specific activity 8 times that of crude platelet extract. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) of this fraction revealed that it consists predominantly of a mixture of high molecular weight proteins (50–200 kDa), suggesting that low molecular weight proteins, including CTAP-III and its derivatives, were not required for enzyme activity (Figure 2). All preparations that exhibited heparinase/heparanase activity contained a prominent 55 kDa band, although the intensity of the stained band had no clear correlation with the level of enzyme activity. It is of interest that fractions "e" and "g" (Figure 2), which exhibited the greatest specific activity (Table 5), contained little protein with a molecular weight > 116 kDa.

Heparin affinity fractionation. Enzymatically active protein fractions from the Sephacryl G-200 column were combined and 0.6 mg of the combined protein mixture was loaded on to a 1 ml HiTrap heparin affinity column equilibrated with 10 mM Na_2HPO_4, pH 6.0. The column was washed with 5 ml of 10 mM Na_2HPO_4 buffer, pH 6, and the bound mate-

Table 3. Ammonium sulfate fractionation of platelet extracts H-7 and H-8, (t = 4–7).

Protein Fraction	Protein, μg/well	Heparin Hydrolysis, %	Total cpm Released	cpm Released/μgProtein (t = 4–7)
H-7, crude extract	15	16	330	22
H-7, 50% supernate	26	0	137	5 (NS)
H-7, 40% ppt	26	17	520	20
H-7, 50% ppt	26	20	572	22
H-8, crude extract	21	19	430	21
H-8, 50% supernate	26	35	1030	40
H-8, 25% ppt	6	22	654	109
H-8, 50% ppt	26	15	440	17

Table 4. Heparinase activity of crude platelet extract and in fractions obtained by gel filtration on Sephacryl G-75.

Protein Fraction	Protein, μg/well	Heparin Hydrolysis, %	Total cpm Released	cpm [^{3}H]-Heparin Released/μg Protein (t = 7–15)
Crude extract (H-13)	35	34	808	15
G-75, fraction 1 (H-13)	6	31	724	121
	13	40	953	73
G-75, fraction 2 (H-13)	6	3 (NS)	69	12

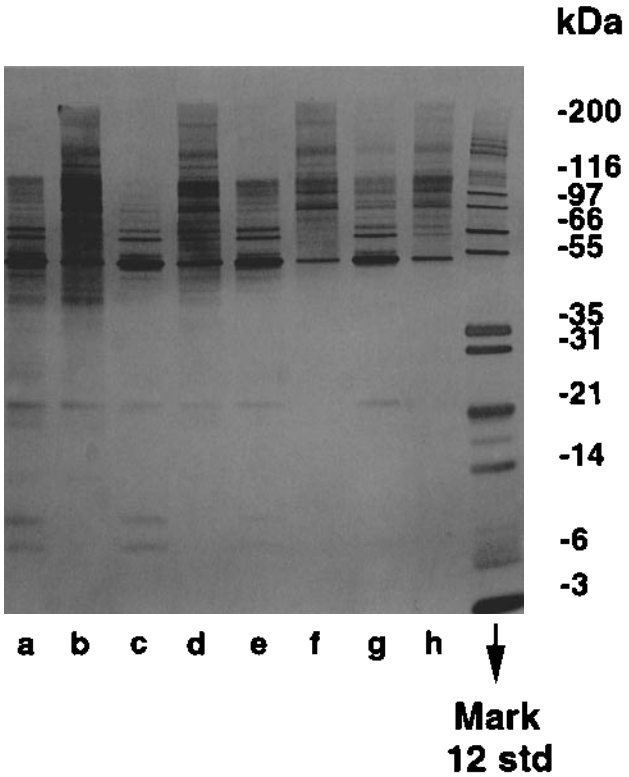

Figure 2. SDS gel comparing crude platelet extract, ammonium sulfate fractions, and fractions from G-75 column. (a) H-13, 40% supernatant; (b) 40% precipitate; (c) 50% supernatant; (d) 50% precipitate; (e) G-75, fraction 1, 40% ammonium sulfate precipitate of fraction; (f) G-75, fraction 1, 40% ammonium sulfate supernatant; (g) 50% ammonium sulfate precipitate of G-75 fraction 1; (h) 50% ammonium sulfate supernatant of G-75 fraction 1. Enzyme activity, expressed as cpm lost/μg protein was 10–12 cpm for samples a–d. CPM lost/μg protein was 76, 45, 97, and 37 for preparations (e) through (h), with the greatest activity shown by sample "g," protein from G-75 fraction 1, which was subsequently precipitated by 50% ammonium sulfate.

rials were then eluted with increasing concentrations of NaCl. Elution from the heparin affinity column was accomplished with sequential concentrations of NaCl at 0.5, 0.75, 1.0, 1.5, and 2.0 M, each adjusted to pH 6.0. The fractions eluted at each salt concentration were pooled, dialyzed against 10 mM Na_2HPO_4 buffer, pH 6.0, concentrated with a Centricon 30 microconcentrator, and tested for heparinase/heparanase activity.

Enzyme activity, shown in Table 6, was restricted to the first 4 fractions eluted from the column, and was greatest in the fraction eluted with 1.0 M NaCl. A second heparin affinity column preparation also showed the major amount of enzyme activity in the 1.0 M NaCl elution fraction. The migration profile of each of these fractions is shown in lanes "b," "c," "d," and "f" (Figures 3 and 4). The molecular weight of the proteins found in each fraction ranged from 22 to $>$ 100 kDa. The most active fraction, "d," was eluted from the heparin affinity column with 1.0 M NaCl and exhibited major bands at 22 and 50 kDa. Lane "d" (shown separately in Figure 4) contains 5 bands ranging in molecular weight from 50 to 100 kDa. In addition, small amounts of the 22 kDa band (discussed above) and 2 bands at the loci of CTAP-III and PF-4 are faintly visible. That the 22 kDa protein was absent in a 0.5 M $(NH_4)_2$ SO_4 fraction (Figure 2, lane "b"), but prominent in the 1.0 M fraction, where both fractions had essentially identical heparanase activity, indicates that the 22 kDa protein is not required for heparanase activity, and that heparanase activity resides in a protein or proteins with molecular weights $\geq$ 50 kDa.

Our initial studies revealed that the CTAP-III immunoaffinity column quantitatively removes CTAP-III and that heparanase activity was not associated with the CTAP-III-rich fraction. To further test CTAP-III and PF-4 for hepari-

Table 5. Heparinase activity in fraction 1 from G-75 and further fractionated with 40% or 50% ammonium sulfate (t = 7–10).

Protein Fraction	Protein, μg/well	Heparin Hydrolysis, %	Total cpm Released	cpm [^{3}H]-Heparin Released/μg Protein (t = 7–10)
H-13, crude extract	70	41	884	13
40% $[NH_4]_2SO_4$ supernate from crude extract	70	36	733	10
40% $[NH_4]_2SO_4$ precipitate	70.0	36	734	11
50% $[NH_4]_2SO_4$ supernate	60.0	33	658	11
50% $[NH_4]_2SO_4$ precipitate	70.0	43	858	12
40% $[NH_4]_2SO_4$ supernate of G-75, fraction 1	6.0	23	467	12
40% $[NH_4]_2SO_4$ precipitate of G-75, fraction 1	11.0	24	474	76
50% $[NH_4]_2SO_4$ supernate	6.0	27	552	97
50% $[NH_4]_2SO_4$ precipitate of G-75, fraction 1	12.0	22	435	37

Table 6. Heparinase activity of platelet proteins isolated by heparin affinity chromatography.

Run 1: NaCl Elution Fraction, M	Protein, μg	Hydrolysis, %	Total cpm Released	cpm/μg Protein (t = 6)
0.50	13.0	15	316	25
0.75	11.0	28	576	53
1.00	7.0	31	641	93
1.50	5.0	18	370	77
2.00	2.0	5	95	NS

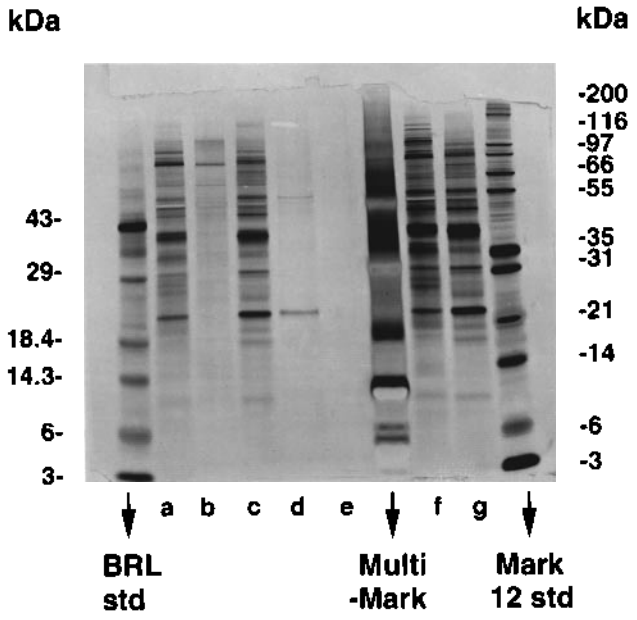

Figure 3. SDS gel showing heparin affinity column fractions on application of proteins from fraction 1 from G-75 Sephacryl column: (a) Sephacryl column fraction 1, 13 μg; (b) heparin affinity column fraction, 0.5 M NaCl; (c) heparin affinity column fraction, 0.75 M NaCl; (d) heparin affinity column fraction, 1.0 M NaCl; (e) heparin affinity column fraction, 2.0 M NaCl; (f) 30,000 g retentate of S-200 fraction 1; (g) 0.75 M NaCl fraction from heparin affinity column.

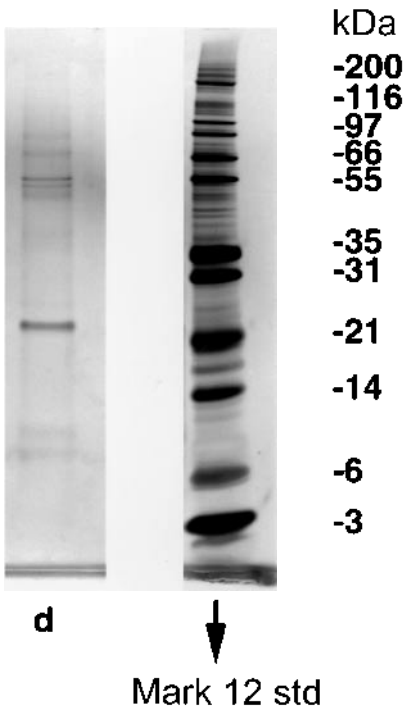

Figure 4. Lane (d) from Figure 3.

nase activity, 2 purified CTAP-III preparations (P-2092 and P-2093) and recombinant PF-4 (Figure 5) were assayed directly for heparinase activity. Concentrations of these proteins ranging from 7 to 21 μg per well exhibited no heparinase activity. Enzymatically active fractions from the heparin affinity column, lane "d," and fraction 1 from the Sephacryl G-200 column, lane "e," are shown for comparison in Figure 5.

The optimum pH for enzyme activity was about 6.0. No activity was detected at pH $\geq$ 7.0.

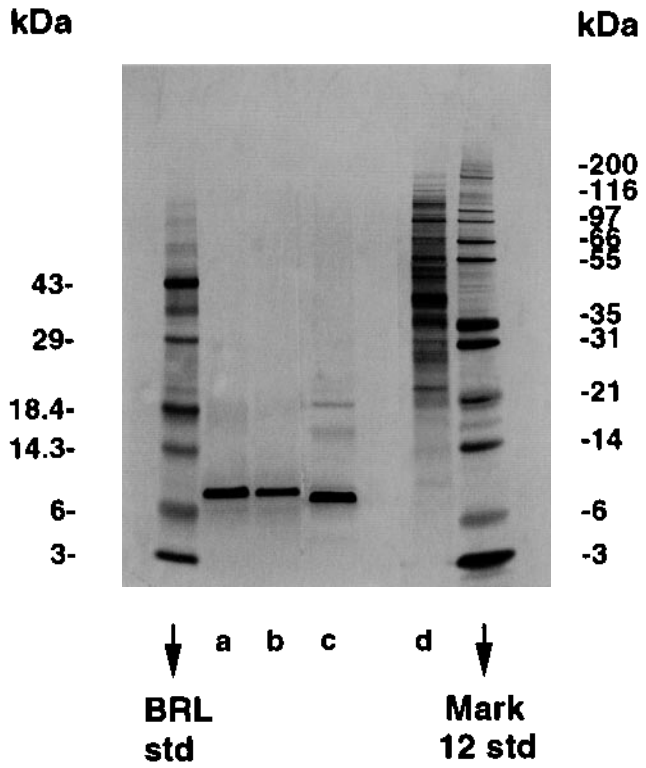

Figure 5. Comparison of the heparinase activity of CTAP-III, PF-4, and high molecular weight fraction from a G-75 column 1: (a) and (b), CTAP-III, preparations 2092 and 2093 had no heparinase activity from 8 to 21 μg protein/well; lane (c), PF-4 had no enzyme activity from 8 μg protein/well; lane (d), 26% hydrolysis from 7 μg protein/well from fraction 1 from S-200 column.

Table 7. Heparinase activity of CTAP-III, PF-4, and a high molecular weight platelet protein fraction from a Sephacryl S-200 column.

Preparation	Protein, μg	Total cpm Removed	Hydrolysis, %
CTAP–III, P-2092	9	0	0
CTAP–III, P-2093	15	4	0
CTAP–III, P-3027	21	0	0
PF-4	8	0	0
Fraction 1 from Sephacryl S-200 column	15	230	16 (t > 4)

DISCUSSION

Oosta, *et al* originally suggested that the molecular weight of human platelet derived heparinase/heparanase was 134 kDa on the basis of PAGE and gel filtration chromatography[7]. A conflicting report[8] more recently assigned platelet heparin/heparanase activity to CTAP-III, a 9278 Da protein characterized and reported by this laboratory[2].

Additional support for the concept that CTAP-III has heparanase activity subsequently came from studies of recombinant CTAP-III prepared from a cDNA library of human CD4+ T cells. This group produced CTAP-III as a fusion protein with a cellulose-binding domain component; it was believed to hydrolyze heparan sulfate, but had no activity against heparin[9].

Data supporting Oosta, *et al*[7] was provided by Freeman and Parish, who described a platelet derived heparinase/heparanase that was shown to have a molecular mass of 50 kDa by gel filtration chromatography and SDS-PAGE[12]. A similar conclusion was reached by Gonzalez-Stawinski, *et al*, who judged the molecular mass to be 35–50 kDa on the basis of size exclusion[11] chromatography and SDS-PAGE. Ihrcke, *et al* provided data indicating that the enzyme activity is associated with a protein whose molecular mass approximates 34 kDa[10].

It may be pertinent, in the context of the discordant results reviewed above, to consider the known biological activities of CTAP-III demonstrated in studies utilizing cell culture[1-4]. The biological activities of CTAP-III include stimulation of DNA synthesis, hyaluronic acid synthesis, sulfated glycosaminoglycan synthesis, glycolysis, prostaglandin E secretion, and intracellular cAMP accumulation by human synovial cells *in vitro*. The magnitude of these various biological activities of naturally occurring CTAP-III isoforms, such as CTAP-III des 1–13, 1–14, 1–15, is increased by as much as 700% compared to the uncleaved form[4]. Recombinant preparations, including rCTAP-III-Leu 21 and rCTAP-III-Leu 21 (des 1–15), also exhibit the same biological activities. CTAP-III des 1–15 (also known as NAP-2) has been shown to activate human neutrophils[5]. Additional biological activities that have been shown for CTAP-III and its isoforms include stimulation of chemotaxis[16] histamine release[17], and glucose transport[18], the latter by increasing the levels of GLUT-1 mRNA.

These findings are clearly consistent with predominantly anabolic and signaling functions of CTAP-III. In view of the anabolic functions of CTAP-III and its derivatives noted above, it is difficult to visualize how a small molecule such as CTAP-III, known to stimulate GAG synthesis, could simultaneously be responsible for the degradation of a particular GAG such as heparin. Consistent with this view, our studies detected no heparinase/heparanase activity in highly purified native CTAP-III, in crude low molecular weight plasma protein fractions containing the CTAP-III, or in recombinant PF-4. In this study, the heparinase/

heparanase activity is limited to platelet protein fractions ranging in molecular weight from 50 to 100 kDa.

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